

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Art Unit : 1635  
Applicant : George F. Vande Woude et al.  
Appln. No. : 10/563,616  
Filing Date : August 9, 2006  
Examiner : Goddard, Laura B.  
Conf. No. : 1900  
For : INHIBITION OF TUMOR ANGIOGENESIS BY COMBINATION OF  
THROMBOSPONDIN-1 AND INHIBITORS OF VASCULAR  
ENDOTHELIAL GROWTH FACTOR

**DECLARATION UNDER 37 C.F.R. § 1.131**

We, the undersigned, do hereby declare as follows:

1. We are the co-inventors of the claims of the above-identified patent application.
2. The invention as defined in claims 1, 5, 7-13, 16-18, 20-22, 26, 28-34, 37, 38, and 40-46 was conceived prior to March 8, 2002, and we were was reasonably diligent in reducing the invention to practice from prior to March 8, 2002, until the filing of our priority application on July 7, 2003.
3. Evidence of our conception and reasonable diligence in reducing to practice the invention as defined in claims 1, 5, 7-13, 16-18, 20-22, 26, 28-34, 37, 38, and 40-46 is provided in the form of experimental data from the laboratory notebooks of Yu-Wen Zhang, one of the named inventors (attached hereto as Exhibit A1-A17). More specifically, these laboratory notebooks show our development of a composition and method for inhibiting tumor angiogenesis comprising TSP-1 and a VEGF inhibitor, including:
  - a) Constructing expression vector pcDNA3/hygro-TSP-1 (Exhibit A1);

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- b) Transfecting SK-LMS-1 cells (SK/HGF cells) with pcDNA3/hygro-TSP-1 to obtain stable ectopic expression of TSP-1 (Exhibit A2);
- c) Amplifying and purifying VEGF DNA fragment for a probe to confirm VEGF upregulation by HGF/SF (Exhibit A3);
- d) Conducting RT-PCR to confirm expression of TSP-1 in stably expressed cell line (Exhibit A4);
- e) Preparing RNA from cells with or without stable expression of TSP-1 (Exhibit A5);
- f) Confirming HGF/SF expression in SK/HGF-TSP-1 cells (Exhibit A6);
- g) Northern blot confirmation of TSP-1 expression in the SK/HGF cells stable transfected with TSP-1 (Exhibit A7);
- g) In vivo mouse experiments to determine the effects of TSP-1 on tumor growth (Exhibit A8);
- h) Preparing RNA from cells treated with inhibitors (Exhibit A9);
- i) Conducting colony formation assays (Exhibit A10);
- j) Northern blot analysis of TSP-1 and VEGF expression (Exhibits A11 and A12);
- k) Northern blot analysis of TSP-1 expression in SK-LMS-1 cells inhibited by MAP kinase inhibitors (Exhibit A13);
- l) Northern blot analysis of VEGF expression in SK-LMS-1 cells inhibited by MAP kinase inhibitors (Exhibit A14);
- m) Preparation of protein lysate from SK-LMS-1 cells treated with MAP kinase inhibitors (Exhibit A15);

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n) Conducting IHC staining of CD31 in tumors derived from mouse study for determining the effects of TSP-1 on tumor angiogenesis (Exhibit A16); and

o) Demonstrating the regulation of VEGF and TSP-1 by HGF/SF in MDA-MB-231 cells (Exhibit A17)


4. The documents attached as Exhibits A1-A17 were prepared contemporaneously with our conception and reasonable diligence in reducing the invention to practice.

5. In the first part of June, 2003, our patent attorney was contacted to begin preparation of related provisional application No. 60/484,676, which was filed in the U.S. Patent and Trademark Office on July 7, 2003.


6. The acts referred to in the preceding paragraphs occurred in the United States.

7. The undersigned hereby declares that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Sections 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

11-21-10  
Date

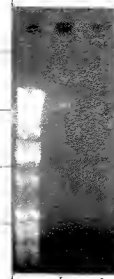
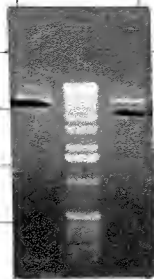
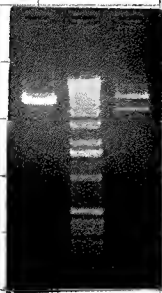
  
George F. Vande Woude

7/21/2010  
Date

  
Yu-Wen Zhang

2/19/02 Construction for pCDNA3.1/Mygro-TSP1.

pCDNA3.1/Mygro (+)	6 $\mu$ l	pCDNA1-TSP1	15 $\mu$ l
10X NE buffer 2	3	10X NE buffer 2	3
10X BSA	3	10X BSA	3
DW	15	DW	6
HindIII	1.5	HindIII	1.5
XbaI	1.5	XbaI	1.5
<hr/>		<hr/>	
30 $\mu$ l		30 $\mu$ l	



gel purified

pCDNA3/Mygro-TSP1

Ligation:

pCDNA3/Mygro (HindIII/XbaI)	0.5 $\mu$ l
TSP1 fragment ( " )	14.5 $\mu$ l
5 x ligation Reaction buffer	4 $\mu$ l
DW	0
T4 DNA ligase	1 $\mu$ l
<hr/>	
20 $\mu$ l	

RT, 1 hour

Transformation:

2  $\mu$ l reaction mixture  $\rightarrow$  1 vial one shot (TSP1)  
twice 30 min  $\rightarrow$  pulse at 42°C for 30 second.

Hygro

2/26/02 Transfection of SK/AGT cells with pCDNA3-Hygro-TSP1

- ① pCDNA3-Hygro vector (1.9  $\mu$ g) + FuGENE 6  $\mu$ l  
② pCDNA3-Hygro-TSP1 (1.9  $\mu$ g)

transfection, 12:40 pm, 2/26/02

2/28/02 split cells for Hygromycin B selection.

1000  $\mu$ g/ml  
600  
500  
1000

3/4/02 Change medium with Hygro

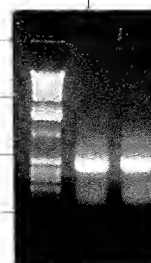
3/7/02 Change medium with Hygro

3/13/02 pick colonies for hygro-control &  
hygro-TSP1

pool stock: SK/AGT-Hygro (800  $\mu$ g/ml, 2ml) - 1 vial  
SK/AGT-TSP1 (800  $\mu$ g/ml, 2ml) - 1 vial

3/6/02 One step RT-PCR for VEGF probe

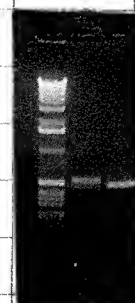
2 x Reaction mix	25 $\mu$ l
Vegf-S (10 $\mu$ M)	1 $\mu$ l
Vegf-as (10 $\mu$ M)	1 $\mu$ l
SK/Hbf total RNA (1 $\mu$ g/ $\mu$ l)	1
DEPC-treated DW	21
RT/PLATINUM Tag mix	1
	50 $\mu$ l



VEGF-L  
VEGF-S



24/line 5000 2500



VEGF-L  
VEGF-S

— use VEGF-as probe

sequencing:

- ① VEGF-L; Vegf-S primer
- ② VEGF-L; Vegf-as primer
- ③ VEGF-S; Vegf-S primer
- ④ VEGF-S; Vegf-as primer
5. pCDNA3-Hygro-TSP1 ③; T7 primer
6. pCDNA3-Hygro-TSP1 ④; T7 primer



2  $\mu$ l DN  
1  $\mu$ l primer  
13.2  $\mu$ l

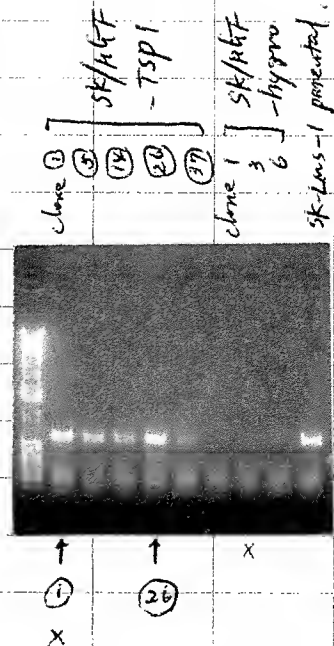
2/15/02 Thawing SK/RTT - TSP clones (1, 5, 14, 26)  
on growing (37).

SK/RTT - hygro clones (1, 3, 6)

2/15/02 RT-PCR to check the expression of TSP1

2 x Reaction mix	12.5
TSP-1 primer (20 µM)	0.5
TSP-2 primer (10 µM)	0.5
RTA sample (1 µg/µl)	0.5
DEPC - DW	10.5
RT/platinum Tag mix	0.5
	<hr/> 25 µl

Method: #20. (21-22-23-24)  
 50°C, 30' } x 1 cycle  
 94°C, 2' }  
 94°C, 15" } x 30 cycles  
 55°C, 30" }  
 72°C, 1' }  
 72°C, 10' - 1 cycle  
 4°C, ∞



8/19/02 Re-isolate RNA for RT-PCR

7

2nd

8/19/02

Eppendorf BioPhotometer 6131 00094  
08/19/2002 V1.01

~~200~~ dilution

17:02 BLANK 0.000 A

17:03 #001 23.0  $\mu\text{g/mL}$  RNA

SK/HLF, Hysno - 1 4.6  $\mu\text{g}/\mu\text{L}$   
0.217 A<sub>230</sub>  
0.575 A<sub>260</sub>  
1.36 260/280 0.422 A<sub>280</sub>  
2.65 260/230 0.001 A<sub>320</sub>

17:05 #002 18.3  $\mu\text{g/mL}$  RNA

SK/HLF, Hysno - 3 3.66  $\mu\text{g}/\mu\text{L}$   
0.172 A<sub>230</sub>  
0.458 A<sub>260</sub>  
1.36 260/280 0.337 A<sub>280</sub>  
2.67 260/230 0.002 A<sub>320</sub>

17:06 #003 13.7  $\mu\text{g/mL}$  RNA

SK/HLF, Hysno - 6 2.74  $\mu\text{g}/\mu\text{L}$   
0.137 A<sub>230</sub>  
0.344 A<sub>260</sub>  
1.35 260/280 0.254 A<sub>280</sub>  
2.51 260/230 0.003 A<sub>320</sub>

17:07 #004 11.8  $\mu\text{g/mL}$  RNA

SK/HLF, TSP - (5) 2.36  $\mu\text{g}/\mu\text{L}$   
0.114 A<sub>230</sub>  
0.296 A<sub>260</sub>  
1.35 260/280 0.219 A<sub>280</sub>  
2.61 260/230 0.002 A<sub>320</sub>

17:09 #005 15.8  $\mu\text{g/mL}$  RNA

SK/HLF, TSP - (14) 3.16  $\mu\text{g}/\mu\text{L}$   
0.153 A<sub>230</sub>  
0.394 A<sub>260</sub>  
1.37 260/280 0.288 A<sub>280</sub>  
2.58 260/230 0.000 A<sub>320</sub>

17:11 #006 20.2  $\mu\text{g/mL}$  RNA

SK/HLF, TSP - (26) 4.04  $\mu\text{g}/\mu\text{L}$   
0.195 A<sub>230</sub>  
0.504 A<sub>260</sub>  
1.37 260/280 0.369 A<sub>280</sub>  
2.59 260/230 0.001 A<sub>320</sub>



10/8/02

RT-PCR for detecting HGF in SK/HGF-TSP1 cells.

		x 10
2 X Reaction mix	12.5	125
HGF-X (10uM)	0.5	5
HGF-Y (10uM)	0.5	5
RNA sample (1ug/ul)	1	
DW (DEPC)	10	100
RT/PLATINUM Tag mix	0.5	5
	25 ul	240 ul → 24 ul/samp + 1 ul RNA

Method: #20 (21-22-23-24)

50°C 30' } x 1 cycle.  
94°C 2'

94°C 15" }  
55°C 30" } x 30 cycles  
72°C 1'

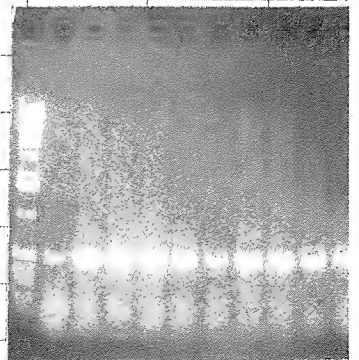
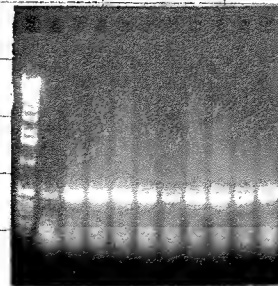
72°C 10' - 1 cycle

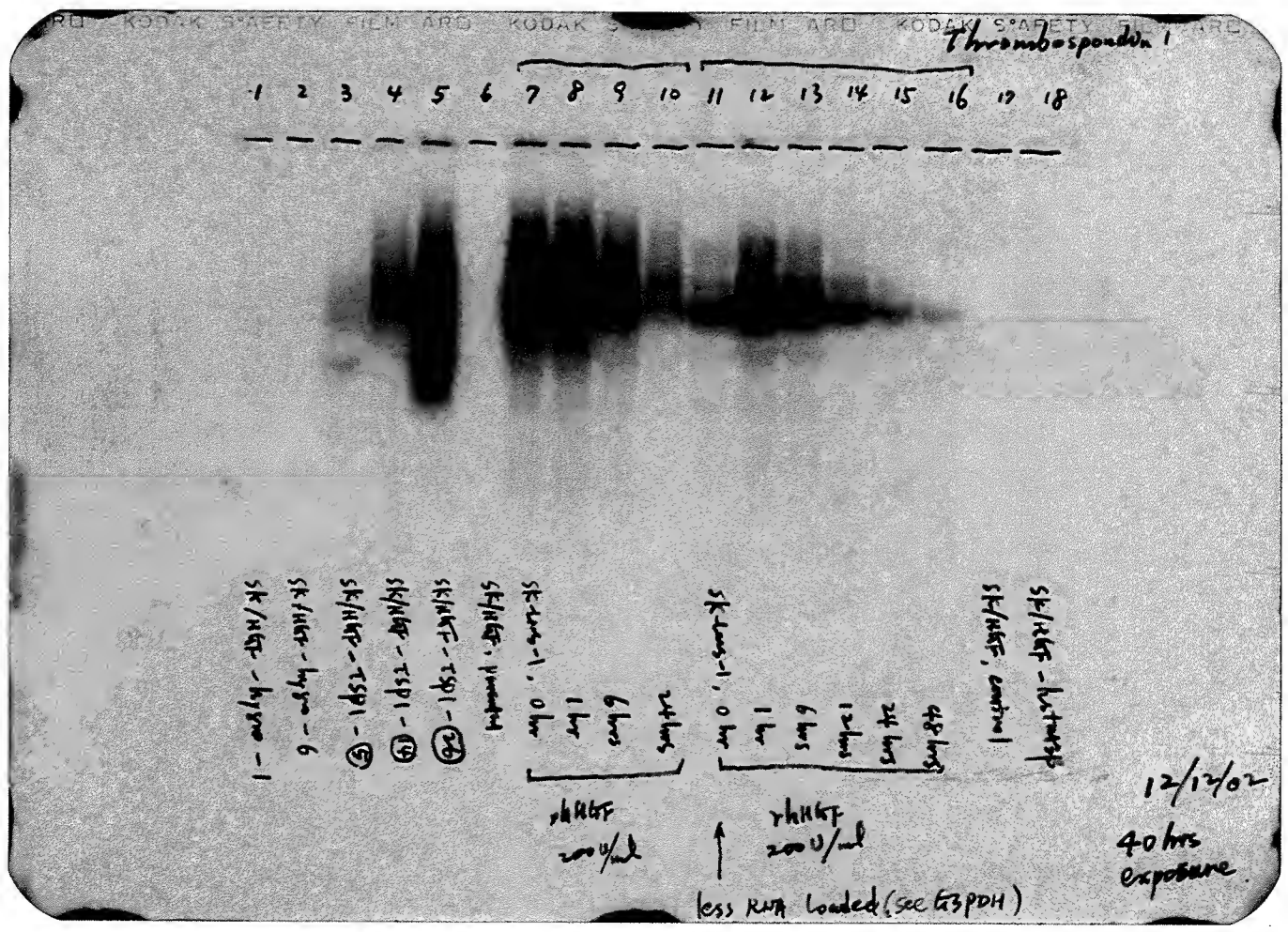
4°C ∞

Samples:

1. SK/HGF-TSP - ①
2. " - ⑤ ✓
3. " - ⑫ ✓
4. " - ⑫ ✓
5. SK/HGF-hygro - 1 ✓
6. " - 3
7. " - 6 ✓
8. SK/HGF parental
9. SK-Lms + parental
10. SK-Lms - 1 parental

①  
⑤  
⑫  
⑫  
hygro-1  
hygro-3  
hygro-6





## Injection of Nude Mice

1/28/2003

5 mice for one group;

$1 \times 10^6$  cells for one mouse;

Prepare cells in the concentration of  $1 \times 10^6$  cell / 100  $\mu$ l in DMEM without serum.

Subcutaneous injection

Samples:

1. SK-LMS-1
2. SK/HGF (SK-LMS-1 autocrine with HGF)
3. SK/HGF-TSP1 (clone 26)
4. C2C12
5. C2C12-hMet (clone 8)
6. C2C12-hMet+hHGF (clone 1)

1. Growing cells in a large flask.
2. Trypsinized cells and pelleted by centrifuge.
3. Resuspending cells in DMEM to the concentration of  $1 \times 10^6$  cells/ 100  $\mu$ l.
4. Injecting nude mice (100  $\mu$ l/mice) by Subcu.
5. Measuring tumor sizes every 3 days.
6. Observing tumors 2-3 weeks after injection.

2/3/03 Treat SK-LMS-1 cells with MAPK and p38 inhibitors in the presence of HGF (200 U/ml) in DMEM + 10% FBS.

		final conc.	Amount / 20ml
1.	DMSO		80 µl
2.	PD98059 (20mM stock)	80 µM	80 µl
3.	U0126 (10mM stock)	40 µM	80 µl
4.	LY294002 (10mM stock)	40 µM	80 µl

2:00pm After treating cells with inhibitors for 1 hour, add HGF. for HGF (100 U/ml stock), add 40 µl to each 20ml medium. incubate for 24 hrs.

2/4/03 isolate total RNA

2:00pm

appendix BioPhotometer 6131 00094  
02/04/2003 1/200 dilution 01.01

17:31 BLANK SK-LMS-1 (+)HGF 0.000 A24 hours

17:33 #001 35.8 µg/mL RNA  
(+) DMSO 7.16 µg/µl 0.343 A230  
0.895 A260  
1.85 260/280 0.642 A280  
2.01 260/230 0.005 A320

17:33 #002 36.6 µg/mL RNA  
(+) PD98059 7.32 µg/µl 0.354 A230  
0.916 A260  
1.40 260/280 0.653 A280  
2.59 260/230 0.000 A320

17:35 #003 42.6 µg/mL RNA  
(+) U0126 8.52 µg/µl 0.416 A230  
1.064 A260  
1.01 260/280 0.757 A280  
2.56 260/230 0.003 A320

17:36 #004 27.5 µg/mL RNA  
(+) LY294002 5.5 µg/µl 0.265 A230  
0.689 A260  
1.38 260/280 0.499 A280  
2.60 260/230 0.001 A320

3/5/03 Colony formation assay in soft agar. (Triplicate)

1. SK-LMS-1 Control cells.
2. SK/HGF Control cells.
3. SK/HGF-TSP1 d(26).

3/19/03 Count colony numbers under phase contrast microscope.  
(size > 0.1 mm; 1000 cells from each plate were counted)

	set 1	set 2	set 3
SK-LMS-1 Control	8	12	19
SK/HGF Control	512	465	538
SK/HGF-TSP1 d(26)	474	525	557

3/18/03

Northern blot

	( $\mu\text{g}/\mu\text{l}$ ) conc.	(10 $\mu\text{g}$ ) RNA	sw up 5.5 $\mu\text{l}$
1. SK/HGF-hygro-6	2.74	3.65	1.85
2. SK/HGF-Tsp1-(26)	4.04	2.48	3.02
3. SK/HGF, parental	5.47	1.83	3.67
4. SK-Lms-1 (no HGF), 0 hr	4.46	2.24	3.21
5. SK-Lms-1 (rHGF), 1 hr	4.34	2.3	3.2
6. SK-Lms-1 (rHGF), 6 hrs	4.6	2.17	3.3
7. SK-Lms-1 (rHGF), 24 hrs	5.26	1.9	3.6
8. SK-Lms-1, rHGF, 1 hr	10	1	4.5
9. " , 6 hrs	10	1	4.5
10. " , 12 hrs	10	1	4.5
11. " , 24 hrs	10	1	4.5
12. " , 48 hrs	10	1	4.3
13. SK/HGF, control	5.47	1.83	3.6
14. SK/HGF - histat3 $\beta$	4.08	2.45	3.0
15. SK-Lms-1 (no HGF), 0 hr	4.46	2.24	3.2
16. SK-Lms-1, rHGF, 24 hrs	10	1	4.5
17. SK-Lms-1 (rHGF, 24 hrs), DMSO	7.16	1.4	4.1
18. " , PD98059	7.32	1.37	4.1
19. " , U0126	8.52	1.17	4.3
20. " , LY294002	5.5	1.82	3.6

Douching:

RNA in

5.5  $\mu\text{l}$ 

10X MOPS

1  
3.5

37% formaldehyde

formamide

10

→ premix  
14.5  $\mu\text{l}$ /sample

EXHIBIT A11

3/21/03 Hybridization with human TSP1.

3/21/03 stripping membrane in <sup>boiled</sup> 500ml of 0.1% SDS solution

↓  
slowly cool down to RT (under 30°C)

↓  
rinse with 2xSSC buffer.

↓  
Rehybridization with human VEGF probe.

3/21/03 Hybridization with human TSP1.

3/21/03 stripping membrane in <sup>boiled</sup> 500 ml of 0.1% SDS solution

↓  
slowly cool down to RT (under 30°C)

↓  
rinse with 2x SSC buffer.

↓  
Rehybridization with human VEGF probe.



1. Sk/Hsp - hygro
2. Sk/Hsp - TSP1
3. Sk/Hsp - powder
4. Sk-Lens-1, no Hsp
5. Hsp, 1 hr
6. Hsp, 6 hrs
7. Hsp, 24 hrs
8. Hsp, 1 hr
9. Hsp, 6 hrs
10. Hsp, 24 hrs
11. Hsp, 24 hrs
12. Hsp, 48 hrs
13. Sk/Hsp, control
14. Sk/Hsp - hot tap
15. Sk-Lens-1, no Hsp
16. Sk-Lens-1, Hsp, 24 hrs
17. DMSO
18. PD96059
19. U0126
20. LY294002

Sk-Lens-1

Sk-Lens-1  
Hsp, 24 hrs

TSP1

40 hrs  
exposure  
(1-3 sec)

1. SK/HGF - hygro
2. SK/HGF-TSP1
3. SK/HGF parental
4. SK-Lous-1, no HGF
5. rHGF, 1 hr
6. rHGF, 6 hrs
7. rHGF, 24 hrs
8. rHGF, 1 hr
9. rHGF, 6 hrs
10. rHGF, 12 hrs
11. rHGF, 24 hrs
12. rHGF, 48 hrs
13. SK/HGF, control
14. SK/HGF - histHGF
15. SK-Lous-1, no HGF
16. SK-Lous-1, rHGF 24 hrs
17. DMSO
18. PD98059
19. U0126
20. LY294002

SK-Lous-1

SK-Lous-1  
rHGF, 24 hrs

V EGF

48 hours

Exposure

3/29/03

3/28/03

SK-LMS-1 treated with inhibitors and/or HGF.

SK-LMS-1 cells were cultured in DMEM + 0.1% BSA for 0/N (Serum starvation).

Add inhibitors:

			HGF (200)
1.	-		-
2.	-		+
3.	DMSO	20ul/5ml	+
4.	PD98059 (10mm stock)	20ul	+
5.	U0126 (10mm stock)	20ul	+
6.	LY294002 (10mm stock)	20ul	+

Incubate cells with inhibitor for 1 hour.

Add 200ul/ml of HGF to each plate.

Incubate for 15 min.

prepare cell extracts in RIPA buffer (with P.I.)  
(in 750ul)

Washing cells three times with ice cold 1xPBS

Add 750ul RIPA buffer to each 10cm dish

Harvest cell lysate using cell scraper and keep in 1.5ml tube

Rotate at 4°C for 15 min.

Freeze in liquid nitrogen for 5 min.

Thaw in ice water

Centrifuge at 13500 rpm, 4°C for 15 min

Collect supernatant and quantify the protein concentration



# rat ABC staining system (Santa Cruz)

4/17/03 Immunohistochemistry staining with rat anti-mouse CD31 antibody

- 1) Formalin-fixed paraffin block section (5 micron) (secondary, anti-rat Ig)
- 2) Deparaffinize :  
3 x Xylene 2 min each  
2 x 100% ethanol  
2 x 95% ethanol
- 3) Block: 1 hour in 1.5% normal serum in 1x PBS
- 4) primary antibody : rat anti-mouse CD31. 1:20 dilution  
incubate at 4°C for o/n in blocking buffer.  
wash three changes of 1x PBS for 5 min each.
- 5) Biotinylated secondary antibody : 30 min at RT.  
wash three changes of 1x PBS for 5 min each.
- 6) AB enzyme reagent : 30 min at RT.  
wash three changes of 1x PBS for 5 min each.
- 7) Peroxidase substrate : add 1-3 drops of peroxidase substrate to each section.  
incubate for 10 min or until desired stain intensity develops.  
wash section in DW for 5 min.
- 8) Hematoxylin counter staining : 10 seconds  
immediately wash several times in DW.
- 9) Dehydrate section (for paraffin-embedded tissue section).  
2 x 95% ethanol for 10 seconds each.  
2 x 100% ethanol for 10 seconds each.  
3 x xylene for 10 seconds each. Wipe off excess.
- 10) immediately add 1-2 drops of permanent mounting medium and cover with a glass coverslip.

5/5/03 Northern blot.

	Concn (1 $\mu$ g/ $\mu$ l)	RNA (20 $\mu$ g)	DW up to 5.5 ml
1. MDA 231 Control -1	10.38	1.93	3.57
2. MDA 231 (+) HGF 24 hrs	11.12	1.8	3.7
3. MDA 231 (+) HGF 48 hrs	9.64	2.07	3.43
4. empty	—	—	—
5. MDA 231 Control -1	10.38	1.93	3.57
6. MDA 231 (+) HGF 24 hrs	11.12	1.8	3.7
7. MDA 231 (+) DMSO (+) HGF 24 hrs	10.96	1.82	3.68
8. MDA 231 (+) PD98059 (+) HGF 24 hrs	7.34	2.72	2.78
9. MDA 231 (+) U0126 (+) HGF 24 hrs	6.8	2.94	2.56
10. MDA 231 (+) LY294002 (+) HGF 24 hrs	7.28	2.75	2.75
11. empty	—	—	—
12. DBTRG Control -1	7.64	2.62	2.88
13. DBTRG (+) HGF 24 hrs	7.66	2.61	2.89
14. DBTRG (+) HGF 48 hrs	10.02	2.0	3.5
15. —	—	—	—

5/6/03 Hybridization with human Tsp1 probe. (exposure: 5/8/03)

5/8/03 Hybridization with human VEGF probe after stripping the membrane (exposure: 5/10/03)

5/13/03 Hybridization with human GAPDH probe (exposure: 5/15/03)